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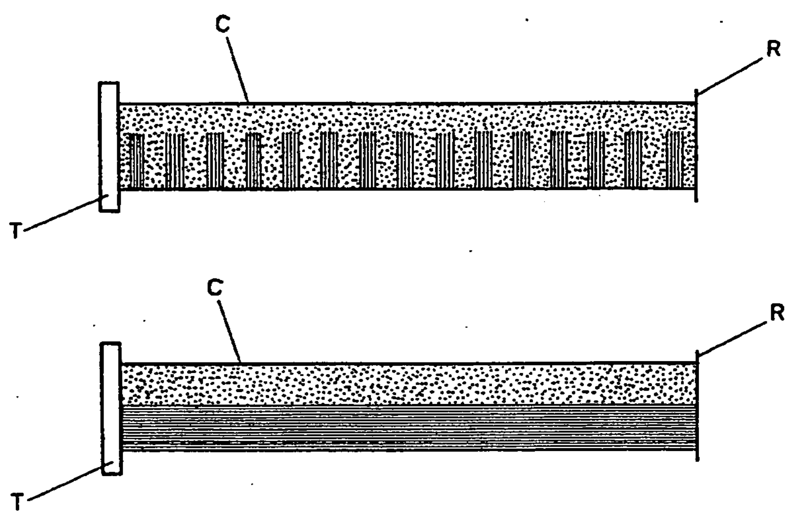

(57) Abstract

In a method of phase partition separation, a mixture of two immiscible liquid phases is subjected to a standing wave ultrasound field, in order to accelerate the separation. The mixture may be contained in a tubular container which may be aligned vertically or horizontally (as shown) or at an inclined angle. The ultrasonic field, which may be pulsed, may extend axially or radially (as shown) of the tubular container. The mixture may include particles which partition into one or other of the two liquid phases.

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Phase Partition Separation Method

This invention relates to a method of phase partition separation of particles.

In this specification, the term "particles" includes particularly, but not solely, cells, bacteria, viruses, organelles and molecules.

Efficient separation and concentration techniques are essential in many areas of biology. Such techniques are required both for soluble substances, e.g. proteins and nucleic acids, and for suspended particles e.g. whole cells, organelles, bacteria and viruses. One well-known separation method used in chemistry is the differential partition of solutes between two immiscible liquid phases, perhaps the most well-known being the ether extraction of impurities in organic preparations. However, organic solvents are not suitable for most biological applications. Nevertheless it is possible to produce two immiscible phases with aqueous solutions of certain polymers: such phases have a water content typically in the range 85-99% (w/w) and are very mild towards various biological activities.

Aqueous two-phase partition was developed by P.A. Albertsson (see 1956 Nature 177:771) for the separation of proteins and other soluble material and adapted for the separation of cells by H. Walter (see 1985 Partitioning in aqueous two-phase systems, Academic Press, London). The two polymers that have been used most extensively for cell separation are polyethyleneglycol (PEG) and dextran. When aqueous solutions of these are mixed together above certain concentrations, two phases are formed, one being PEG-rich and the other dextran-rich. When particles are present in the system they distribute or partition, often principally between the interface and one of the bulk phases, thus enabling highly selective separations to be achieved.

Phase separation of biological particles is a two-step process with each step having different characteristic times. The first stage is the partition of the particles or solute

into drops of one of the phases. This step is rapid. The second stage is the separation of the phases into discrete layers allowing collection of the particles or solute. It is this stage which can take a considerable time to occur. It has been known for some time that particles or droplets in a stationary ultrasonic field can experience time-independent forces that move them to preferred regions of the field separated by half wavelength intervals. A number of techniques involving single or double transducer systems or stationary or moving reflectors have been proposed for moving ultrasonically concentrated small particles or large single particles in suspension in order to achieve efficient particle harvesting. However, ultrasonic manipulation of very small (sub-micron sized) particles is difficult because the radiation force acting on the particle is proportional to particle volume. The speed at which particles can be harvested from suspension by ultrasonic forces is also particle-size dependent so that small ($10\mu m$ - $1\mu m$) particles can best be harvested from suspension when they are at such a concentration as to allow initial particle collection into clumps (which will then behave as the equivalent of large particles) at positions close to either pressure minima or pressure maxima throughout the standing wave field.

We have now devised a method which accomplishes phase partition separation within a reduced time period.

In accordance with this invention, there is provided a method of phase partition separation, in which a mixture of two immiscible liquid phases and particles is subjected to a standing wave ultrasound field.

We have found that, in use of this method, the coalescence of droplets and development of separate bulk phases is considerably accelerated.

The ultrasound field may extend in any convenient direction through the mixture or phase system. Conveniently, the field may extend either axially or transverse of a tube or other elongate container in which the phase system is contained: for example the container may be positioned with its axis generally vertical or generally horizontal, or at an inclined angle between horizontal and vertical if desired. In

order to produce a field transverse to the axis of the container, an annular or tubular transducer may be used, preferably with the transducer also forming a tubular wall of the container: in this case the ultrasound field is a radial field.

The applied ultrasound field causes the phase system to form into a series of bands of the two phases, separated by a half wavelength. Preferably the ultrasound field is pulsed: when the field is interrupted, the bands of the more dense phase fall under gravity: some break-up of the bands occurs before the field is re-applied.

The method may also be used simply for separating two liquid phases, e.g. separating one liquid from another in an emulsion. Therefore, also in accordance with this invention, there is provided a method of phase partition separation in which a mixture of two immiscible liquid phases is subjected to a standing wave ultrasound field.

Embodiments of this invention will now be described by way of examples only and with reference to the accompanying drawings, in which:

Figure 1a is a schematic diagram of a single transducer arrangement for carrying out methods in accordance with this invention;

FIGURE 1b is a similar diagram of a dual transducer arrangement;

FIGURE 1c is a similar diagram of an annular transducer arrangement;

FIGURE 2 is a graph to compare the times taken for the formation of two continuous bulk phases under gravity and under the influence of ultrasound, for phase systems of varying relative proportions;

FIGURE 3 is a diagrammatic side view of the arrangement of Figure 1a when aligned vertically, showing the separation of phases into bands when subjected to an axial ultrasound field;

FIGURE 4 is a diagrammatic side view of the arrangement of Figure 1a to show the degree of separation of the mixture into two phases at successive times of a) 0 min, b) 1 min, c) 2 min and d) 3 min;

FIGURE 5 is a diagrammatic side view of the arrangement of Figure 1a when the tubular container is positioned horizontally, showing a) banding of the phases after 30s and b) complete separation of the phases after 90s;

FIGURE 6a is an end view of the annular transducer of Figure 1c, showing formation of annular zones under the influence of a radial ultrasonic field produced by the transducer; and

FIGURE 6b is a similar view of the annular transducer when positioned with its axis horizontally, at complete phase separation.

Figure 1 shows schematically three different arrangements which we have used by way of examples for carrying out methods in accordance with this invention. In Figure 1a, a standing wave field is generated in a cylindrical container C using a single transducer T and a reflector R at its opposite ends: Figure 1b shows the use of two transducers T at the opposite ends of the container C. The or each transducer is driven by an amplifier A (Model A150,ENI, Rochester N.Y.), the input for which is provided by a Hewlett Packard 3326A two-channel frequency synthesizer S. The transducers T are 40 mm diameter air-backed piezoceramic discs, driven at their fundamental thickness resonance of 1 MHz. In some cases we reduced the diameter of the rear electrode to 8.4 mm in order to excite primarily the lowest, axially symmetric (Bessel shaped) mode of the waveguide. A transducer with this electrode pattern would be expected to produce a Gaussian field in an unbounded liquid. Any temperature changes induced by ultrasound treatment were measured using a 4.7 kΩ thermistor. In the arrangements of Figures 1a and 1b, the ultrasonic standing wave field is generated axially of the container C. Figure 1c shows an arrangement in which a radial ultrasonic field is generated using an annular transducer T. Ultrasound was generated from a 32 mm internal diameter, 12 mm long tubular air-backed piezoceramic transducer T with a 665 kHz radial thickness resonance. The transducer T was enclosed between two discs of perspex having ports made from shortened (3 mm) 19 gauge syringe needles which did not protrude into the sound field: the transducer T and these two discs formed the

container for the mixture.

By way of examples, organisms were grown as follows, for use in the arrangements shown in Figure 1. Thus, *Saccharomyces cerevisiae* 370.1D was grown in 200 ml of YEPD medium until late exponential phase ($O.D_{600}$ = 3-4). Cells were incubated overnight at 30°C with shaking at 2Hz. Cells were harvested and washed twice in 50 ml quantities of 10 mM Tris.HCl, pH 7.6 followed by fixation in 3% glutaraldehyde for 1h. Cells were washed in 50 ml of 10 mM Tris.HCl, pH 7.6 and stored in the same buffer. Yeast cells were fixed in glutaraldehyde solely to ensure a ready supply of cells. A number of comparative experiments failed to reveal a detectable difference in the partitioning of fixed and unfixed cells or in their subsequent behaviour when the phase system was exposed to ultrasonic fields. Also, *Escherichia coli* NCTC 10418 was grown in 200 ml nutrient broth (Oxoid). Cells were incubated at 37°C with shaking at 2 Hz. Cells were used live directly from the flasks at $O.D_{600}$ =0.3-0.5, *i.e.* mid-to-late exponential phase.

Further by way of example, phase systems were produced by mixing equal volumes of 10% (w/v) 500 kD dextran (Pharmacia) and 10% 8 kD (w/v) PEG (Sigma Ltd.) to give a final concentration of 5% (w/v) for each polymer. Where yeast or bacteria were used, these were added at approximate concentrations of 10^7 cells ml^{-1}. 1.0 ml of cell suspension was added to 10 ml of the phase system, resulting in a modified polymer concentration of 4.55% (w/v) for each polymer.

Samples of the phase systems were diluted 1:10 with 10 mM Tris.HCl (ph 7.6). Cell numbers were counted directly (through light scattering at 436 nm from individual cells) in an Argus flow cytometer (Skatron, Norway). The sample flow rate was set at 1 μl/min. The time to count 10,000 cells was determined for bacteria. In the case of yeast the accumulated cell count at the end of 5 min was noted.

In order to compare phase partition under gravity and ultrasound, we carried out initial experiments involving measurements of the time required for phase separation when the simple PEG/dextran system was separated under gravity or under the influence of an applied acoustic field. A standing wave

ultrasonic field was applied to freshly mixed samples of the phase system contained in an acoustic chamber C in the arrangement illustrated in Figure 1a. Alternate layers of a dextran-rich and a PEG-rich solution were formed in less than 1 min at transducer voltages in excess of 2 Vp-p and 3 Vp-p for 4 ml and 10 ml containers respectively. The layers formed within 3 s at voltages of twice the above threshold values. If these supra-threshold voltages were maintained on the transducer, layers of the separate phases could be maintained for long time periods (> 1 hr). Settling of some (10-20%) of the heavier phase could occur over a 1 hour time period. When the power was turned off, the heavier dextran layers settled under gravity. However, during the settling process some fragmentation of the bands occurred and there was a degree of remixing of the phases. The application of repetitive pulses of sound layered the remixed sections and led, during the "sound off" period, to growth of the heavier phase as a continuous volume at the bottom of the container. In initial experiments the phase systems were subjected to pulses of ultrasound of 30 s duration followed by 30 s with no sound. The time for formation of two continuous phases was measured and compared to the time required for separation under gravity, for different relative proportions of the phases: the results are shown in Figure 2. The amount of each polymer was kept constant for all of the systems. Figure 2 illustrates a significant reduction in the time taken for separation under the influence of ultrasound compared with settling under gravity alone. It was not possible to prepare initial concentrations of PEG capable of giving a final concentration of 5% (w.v) if the mixture of the initial PEG volume was less than 10% of the total volume. This observation is in accordance with theoretical partition diagrams (see P.A. Albertsson 1986; Partition of cells, particles and macromolecules, Wiley, New York). The above investigations showed that phase separation can be achieved in an orderly manner using pulsed ultrasound to rapidly form layers of the two phases and then allow the layers to settle under gravity during the "sound-off" periods.

We further used the arrangements of Figure 1a for the

phase partition of yeasts and bacteria. Of the two microorganisms used, *Saccharomyces cerevisiae* partitions into the dextran phase, whereas *Escherichia coli* partitions into the PEG phase. When a standing wave was applied to the PEG/dextran system, characteristic bands of PEG-enriched and dextran-enriched layers were formed. When organisms were also present in the cylindrical acoustic chamber, they appeared in the appropriate bands P or D as shown in Figure 3. Thus in the case of *S. cerevisiae*, when the voltage was turned off the yeast cells contained in bands and drops of dextran sedimented to the bottom of the acoustic chamber. Conversely *E.coli*, which partitions into the PEG enriched phase, separated towards the top of the vessel.

In order to optimise the timing of the phase separation by pulsed ultrasound, we tested various combinations of sound on, and sound off, in the time range of 1s to 1 min. Settling times (sound off) lower than 5 s resulted in the layers being reformed close to their original positions when the transducer voltage was reapplied, with very little development of continuous bulk phases occurring. The optimum time for separation was achieved with 7.5 s pulses of sound, followed by 7.5 s settling time. This regime produced complete separation (shown in Figure 4) in 135 s for 4 ml volumes. The temperature increase over this period was <1°K at the working voltage of 4.4 V p-p.

One of the limiting steps in reducing the time of separation is the remixing of the phases which occurs during the settling period. In order to reduce these remixing effects, we made a number of tests on acoustic chambers which were inclined to the vertical. However, operation of the system at angles of approximately 45° did not result in any improvements with regard to the time of partition (Table I). When cylindrical vessels were used horizontally (*i.e.* inclined at 90°, as shown in Figure 5) much reduced partition times were obtained. Thus, 10 ml of the phase system could be separated in 75 s. The reduction in remixing of the phases is partly due to the reduction in the distance through which the heavier phase falls when settling under gravity.

We also used the arrangement of Figure 1b with the

phase system (4.5% PEG/4.5% dextran / 10^7 yeast cells per ml), using a 10 cm long vertical tube as the container C, held between two transducers T driven at a frequency of 1.023 MHz and an applied voltage of 7.2 V p-p. When bands of the phase system containing *S. cerevisiae* were established, the frequency of one transducer was altered by a selected increment in the range $\pm$ 1 Hz to $\pm$ 3 Hz. The bands containing the microorganisms moved upwards or downwards towards the transducer driven at the lower frequency. The increment of 1 Hz allowed us to obtain phase separation of yeasts in approximately 70 s.

We further used the arrangement of Figure 1c for exposing the PEG/dextran system containing *S. cerevisiae* to a radial ultrasonic field. In the case of annular transducers, generally the pressure maxima and minima produce tubular zones of the two phases rather than bands, as shown in Figure 6a. The annular transducer was mounted in a horizontal position (axis vertical) so that the rings of dextran containing *S. cerevisiae* would drop to the bottom of the vessel when the sound was turned off, with a much reduced remixing of the two phases. This appeared to be the case but was difficult to confirm visually due to the opaque wall of the transducer, therefore timings for separation were based on microscopic examination of two samples taken 1 mm from the top and 1 mm from the bottom of the vessel respectively (samples were removed with a syringe attached to a micromanipulator). Separation was assumed to be complete when the top sample was essentially devoid of cells and the concentration of cells in the bottom phase reached a maximal value. When we exposed the phase system to two 30 s pulses of ultrasound separated by 15 s, separation appeared to be complete within the total period of 90 s.

We also used a tubular transducer in the vertical position (axis in the horizontal plane). When ultrasound was applied to a phase system containing *S. cerevisiae* rings composed of dextran enriched regions containing cells were clearly visible, as shown in Figure 6a. When the sound was turned off separation into continuous bulk phases occurred, as shown in Figure 6b. However, in order to accelerate this

process we again pulsed the sound. A regime of 7.5 s pulses followed by 7.5 s with no sound resulted in phase separation of *S. cerevisiae* in 75 s. The increase in temperature over this period was <1°K when the transducer voltage was 6.8V p-p (almost 3 times the threshold voltage of 2.4 V p-p).

The times required for ultrasonic separation of two continuous bulk phases are compared in Figure 2 with the times required for the separation of continuous phases under gravity. The times for phase separation (4.55% w/v PEG, dextran system containing cells) under the faster sedimentation rates obtainable in a bench centrifuge (10 ml sample in a test tube exposed to 300 g) were also measured (Table I). The Table also contains the measured partition "yields" of cells in the preferred phase for centrifugation-enhanced and for ultrasound-enhanced systems. The data shows that ultrasound compares well with centrifugation both in terms of timing and of partition "yield". The times for partition of *E. coli* into continuous bulk phases were as the times for *S. cerevisiae* separation. The partition "yields" for *E. coli* were, on average, 1.6% (±0.25% 95% confidence limits) lower than those for yeast (Table 1). For the results reported in Table 1, the transducer voltages for the different systems were in the range 2.2 to 2.6 times the threshold voltage for layer formation in 1 min. The yields were calculated from the percentage of cells in the PEG phase (based on bacterial counts of 10,000 and yeast counts in the range 10-200). There was good agreement between triplicated counts. Each experimental arrangement was tested twice. While the bacterial partition "yield" was consistently lower than the yeast yield, the mean of the four values (in the last column) gives summary guidance to the performance of the different systems.

The radiation force F_r exerted by a plane standing wave of peak pressure P_0 and wave number k on a droplet of volume V, density ρ^* and compressibility β^* suspended in a fluid of density ρ and compressibility β is given by:

$$F_r=[-VP_o^2k/4][(5\rho^*-2\rho)/(2\rho^*+\rho)-\beta^*/\beta]\sin 2kz \text{--------------} (1)$$

where z is distance at right angles to the planes of iso-pressure. Similar expressions describe the force on particles in non-plane standing waves. The sine term in Eqn. 1 leads to the distribution of stable resting positions or droplets separated by half an acoustic wavelength. There is, in addition an inter-droplet force F_s, which is given for droplets of equal radius, R, by:

$$F_S=R^6[P^2_{max}.f(\beta^*,\beta)/L^2+U^2_{max}.f(\rho^*,\rho)/L^4] \text{-------------} (2)$$

where L is distance between the droplet centres, P_{max} and U_{max} are the local maximum acoustic pressure and velocity amplitudes respectively. Expressions for the compressibility function and the density function terms of Eqn. 2 can be derived, respectively from R.E. Apfel (1990 Tagungsbandzur 16, Gemeinschaftagung der DAGA, Teil B, pp. 19-36) and from W.L. Nyborg (1976 In; Fry, F.J., Ultrasound: Its application in medicine and biology, Pt 1 Elsevier, Amsterdam pp. 1-75).

Our investigations suggest that the dextran (yeast containing) phase acts as a dispersed phase in a continuous PEG enriched phase. The rapid (<3 s) development of layers of each phase suggests that dextran droplets, growing by coalescence due to inter-droplet acoustic forces (Eqn. 2), migrate (Eqn. 1) to regions of the standing wave field where droplet concentration occurs and the probability of coalescence therefore increases.

Table 1 shows that the completion time (*ca* 70 s in the tubular transducer) for bulk phase separation of *S. cerevisiae* by ultrasound is faster than that (3.5 min) achieved by centrifugation of the phase systems. Differences between the mechanisms of development of two final bulk phases by ultrasound and by centrifugation include that while the "g" forces exerted by the sound field are generally lower than those achievable by centrifugation, the distances which droplets need to move before forming a layer in a sound field are very short (a quarter wavelength is 0.37 mm at 1.0 MHz) compared with the length of a centrifuge tube. Additionally there is no centrifugation equivalent of the acoustic interdroplet force described by Eqn. 2.

The times required for final bulk phase development for the different field geometries (plane and tubular transducers) at different inclinations to the vertical vary from 5 min to 1.2 min for volumes of the order of 10 ml (Table I). The Table also shows that the horizontally-positioned 10 ml cylindrical tube and the 12 ml tubular transducer give high partition "yields" most rapidly. The horizontal cylinder (Fig. 5) and the tubular transducer with its axis vertical clarify rapidly because the distance through which the layers sediment is short and the geometry allows layers to sediment or rise with little intermingling, thus reducing layer fragmentation. However, the system which offers particularly rapid sedimentation and a simple means of recovering the phase appears to be a tubular transducer with its axis horizontal (Fig 6). The lower percentage of cells (compared to centrifuge-enhanced separation) remaining in the non-preferred phase in those ultrasonic systems which were particularly rapid and had high partition "yields", suggest that these systems were particularly efficient in removing dextran-enriched droplets from the PEG bulk phase (Table I). The low temperature rises recorded (<1°K under partitioning conditions) and the relatively low transducer voltages needed testify to the low power levels required for the ultrasonic partitioning.

In addition to the separation of cells or other biological particles into a single phase as described above, aqueous two-phase partition has been employed to fractionate biological preparations through repeated phase separations (P.A. Albertsson, 1986; Partition of cells, particles and macromolecules, Wiley, New York): currently the process for achieving this fractionation involves countercurrent distribution techniques which require successive repartitioning of fractions e.g. by centrifugation. The more rapid separation achievable in accordance with this invention, without moving parts, with an array of transducers has the potential to make two phase partition techniques more practical.

SYSTEM	CONTAINER	VOLUME (ml)	TIME (min)	YEAST		CELL YIELD (%) BACTERIA		AVE
Centrifugation: (300 g Bench Centrifuge)	Centrifuge tube	10	3.5	97.4	98.8	97.3	96.3	97.5
Ultrasound, Disc Transducer: (4.4 V_{p-p}: pulsed 7.5 s on, 7.5 s off)	Vertical cylindrical tube	4	2.2	99.0	99.4	97.7	97.5	98.4
(7.2 V_{p-p}: pulsed 7.5 s on, 7.5 s off)	Vertical cylindrical tube	10	5.0	97.5	98.1	96.4	95.8	97.0
(4.4 V_{p-p}: pulsed 7.5 s on, 7.5 s off)	Inclined (45°) cylinder	4	2.2	99.1	98.5	96.6	97.0	97.8
(7.2 V_{p-p}: pulsed 7.5 s on, 7.5 s off)	Inclined (45°) cylinder	10	5.0	97.3	98.9	96.2	97.0	97.4
(7.2 V_{p-p}: pulsed 7.5 s on, 7.5 s off)	Horizontal cylinder	10	1.5	99.5	99.1	97.4	97.0	98.2
Ultrasound quasi-standing wave	Vertical cylinder	4	1.2	98.5	95.9	NA		
(2 Transducers; 1 Hz increment)	Vertical cylinder	10	1.5	95.9	96.9	NA		
Ultrasound; Tube Transducer: (6.8 V_{p-p}, 7.5s on; 7.5s off)	Tube axis vertical	12	< 1.5	98.7	98.9	97.8	97.4	98.2
	Tube axis horizontal	12	1.2	99.2	99.0	98.1	97.5	98.4

TABLE 1.

CLAIMS

1) A method of phase partition separation, in which a mixture of two immiscible liquid phases and particles is subjected to a standing wave ultrasound field.

2) A method of phase partition separation in which a mixture of two immiscible liquid phases is subjected to a standing wave ultrasound field.

3) A method as claimed in claim 1 or 2, in which the ultrasound field is pulsed.

4) A method as claimed in any preceding claim, in which the ultrasound field is directed substantially vertically.

5) A method as claimed in any one of claims 1 to 3, in which the ultrasound field is directed substantially horizontally.

6) A method as claimed in any one of claims 1 to 3, in which the ultrasound field is radial.

7) A method as claimed in claim 6, in which the axis of the radial ultrasound field is generally vertical.

8) A method as claimed in claim 6, in which the axis of the radial ultrasound field is generally horizontal.

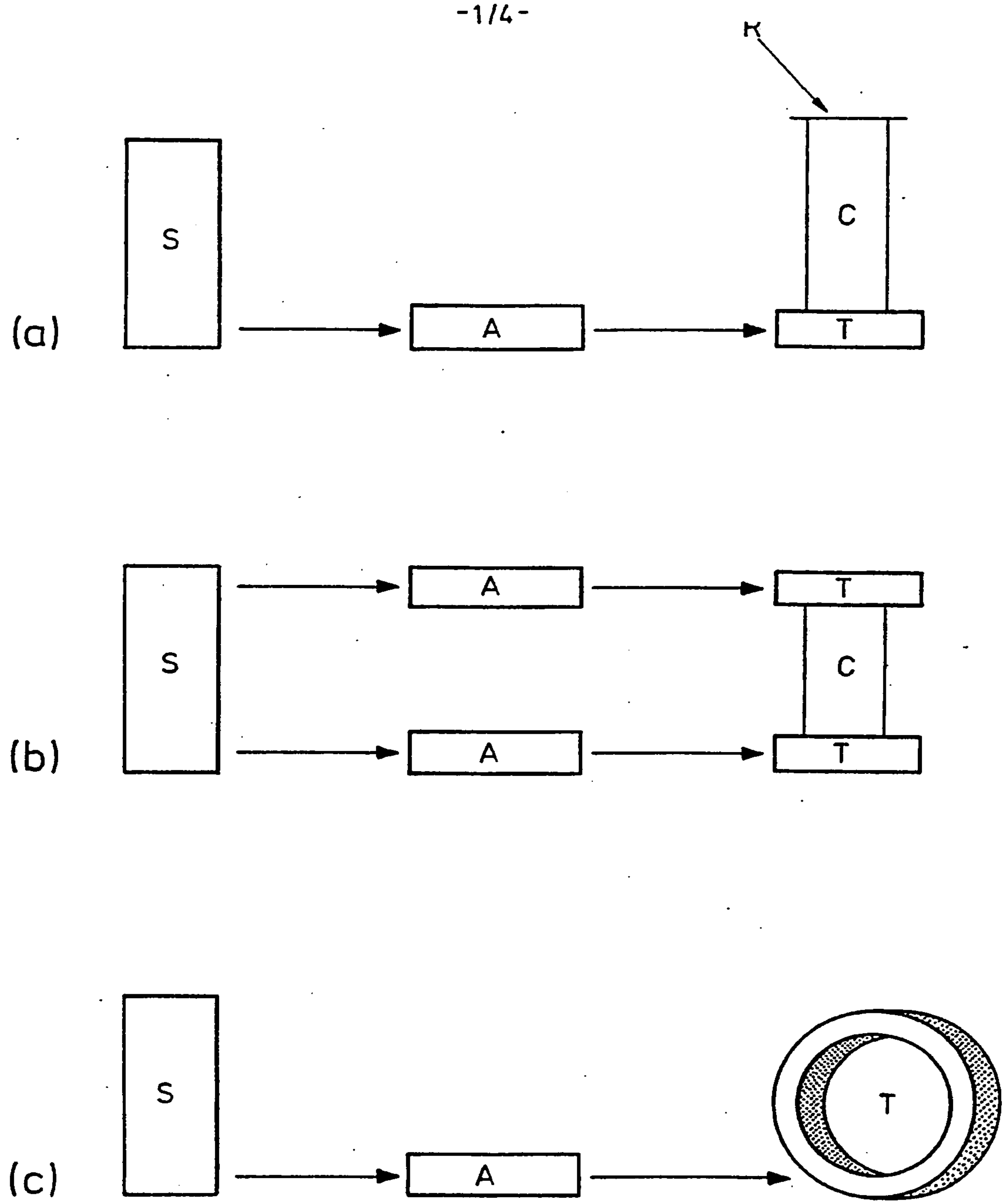

FIG. 1

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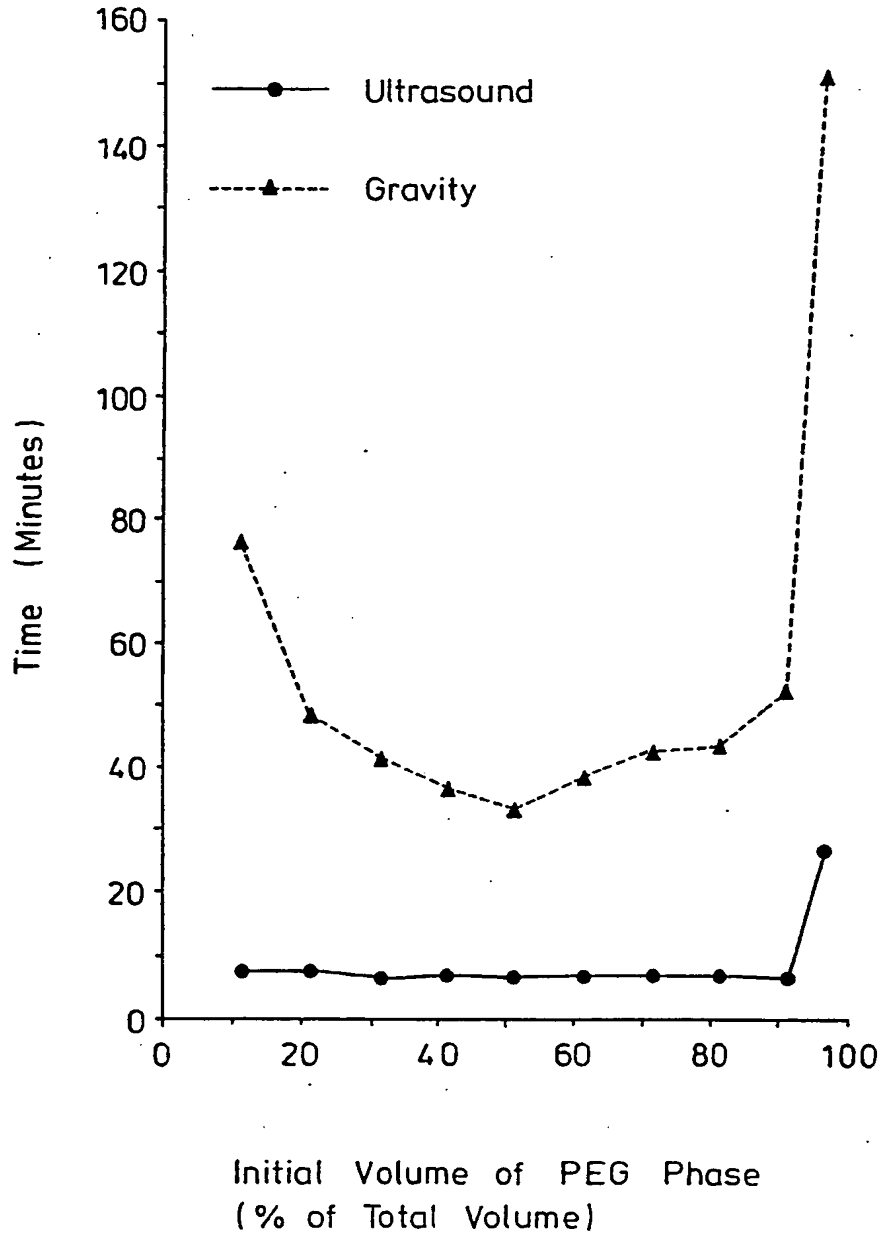

FIG. 2

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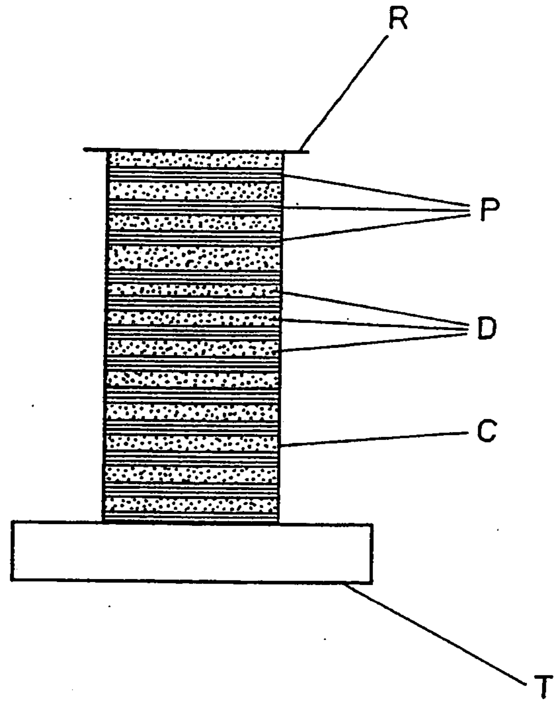

FIG. 3

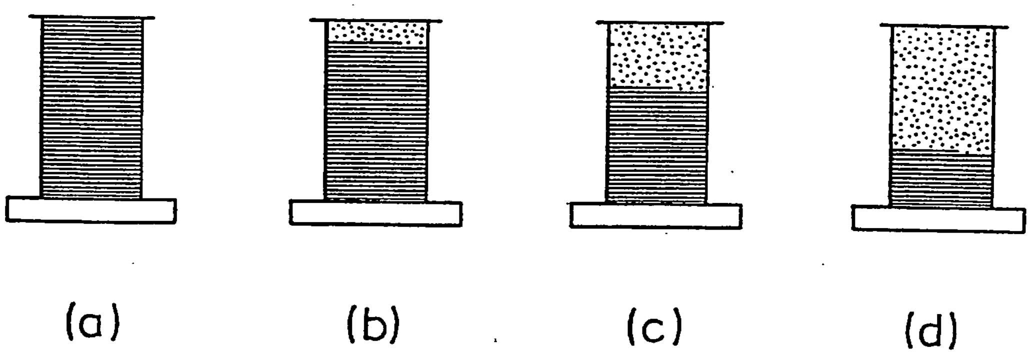

FIG. 4

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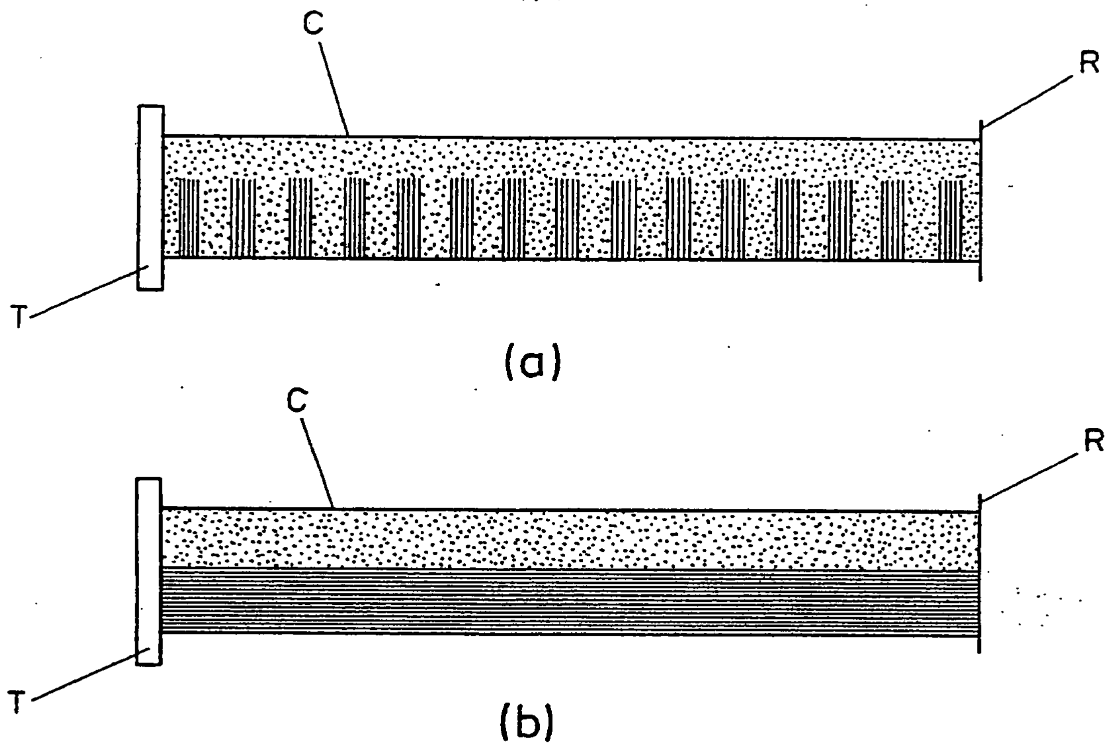

FIG. 5

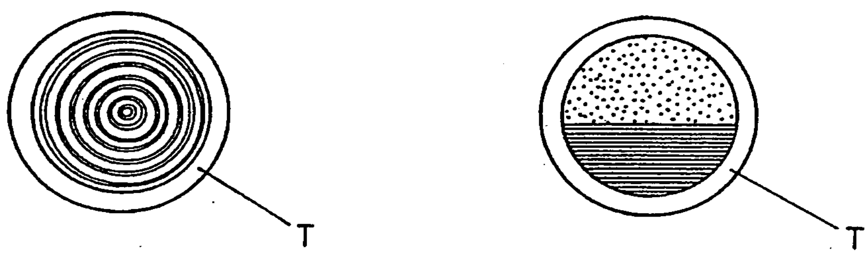

FIG. 6

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INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 93/01315

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 G01N33/00; B01J19/10; B01D17/04; B01D21/28

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	B01D ; B01J ; G01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category°	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	US,A,3 594 314 (BILHARTZ ET AL.) 20 July 1971	1,2
Y	see column 1, line 13 - line 33	3-8
X	CHEMIE INGENIEUR TECHNIK vol. 55, no. 9, September 1983, NURNBERG, DEUTSCHLAND pages 700 - 707 KRIECHBAUMER ET AL. 'HERSTELLUNG, STABILITÄT UND SPALTUNG MULTIPLER EMULSIONEN'	1,2
Y	EP,A,0 292 470 (STUCKART) 23 November 1988 see column 1 - column 2; figures	3-8
X	DE,C,886 884 (SIEMENS-SCHUCKERTWERKE AG) 17 August 1953	2
A	see claim 1; figures	1,3-8

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° Special categories of cited documents :[10]

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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"&" document member of the same patent family

IV. CERTIFICATION

1

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 OCTOBER 1993	29. 10. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MARZENKE J.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
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This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82